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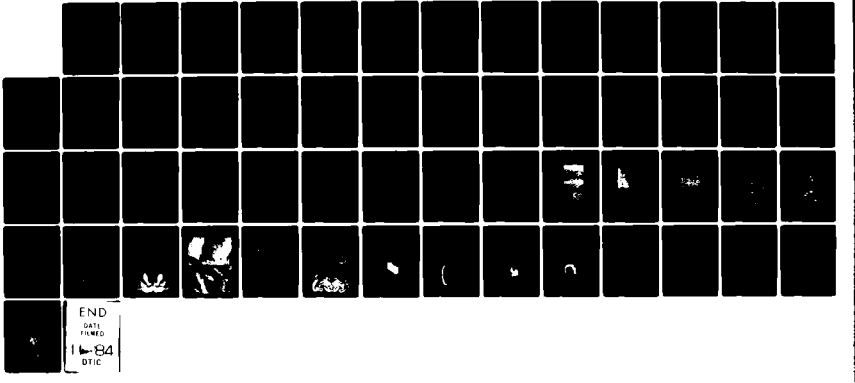

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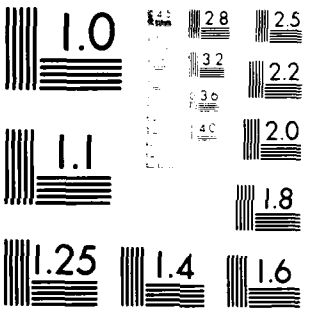

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striate cortex, indicating that this innervation arises entirely from an extrinsic source in the cat. To identify this source, cell groups projecting to area 17 were retrogradely labelled with horseradish peroxidase (HRP). HRP-labelled cell groups that were also intensely AChE-positive were considered as possible candidates for providing the cholinergic input to striate cortex. These included the basal forebrain, several intralaminar nuclei and the lateral geniculate nucleus. Kainate lesions were then made in each of these structures to assess their individual contributions to the cortical AChE pattern. Cortical AChE was depleted only after lesions of the basal forebrain, suggesting that this is the sole source of AChE-positive axons in area 17. Because the cortically-projecting cells in this region have been shown to contain choline acetytransferase in a number of species, we postulate that the AChE-positive fibers we describe in cat striate cortex are in fact cholinergic.

# AN INVESTIGATION OF CHOLINERGIC CIRCUITRY IN CAT STRIATE CORTEX USING ACETYLCHOLINESTERASE HISTOCHEMISTRY

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## ABSTRACT

The organization of cholinergic inputs to cat striate cortex (area 17) was studied using a histochemical stain for acetylcholinesterase (AChE). Axons were labelled in all layers of striate cortex, with distinct plexuses occurring in layer I, lower layer III, layer IVc and layer VI. In addition to the stained axons, a population of layer V pyramidal cells was intensely AChE-positive.

Surgical undercutting eliminated virtually all of the AChE-positive axons in striate cortex, indicating that this innervation arises entirely from an extrinsic source in the cat. To identify this source, cell groups projecting to area 17 were retrogradely labelled with horseradish peroxidase (HRP). HRP-labelled cell groups that were also intensely AChE-positive were considered as possible candidates for providing the cholinergic input to striate cortex. These included the basal forebrain, several intralaminar nuclei and the lateral geniculate nucleus. Kainate lesions were then made in each of these structures to assess their individual contributions to the cortical AChE pattern. Cortical AChE was depleted only after lesions of the basal forebrain, suggesting that this is the sole source of AChE-positive axons in area 17. Because the cortically-projecting cells in this region have been shown to contain choline acetyltransferase in a number of species, we postulate that the AChE-positive fibers we describe in cat striate cortex are in fact cholinergic.

## INTRODUCTION

In 1967 Shute and Lewis mapped the central cholinergic pathways using a histochemical stain for acetylcholinesterase (AChE). The AChE-containing pathways of the forebrain seemed to be derived largely from cells in the reticular formation of the brainstem and included cell groups in the intralaminar thalamus and basal telencephalon. They called this the "ascending cholinergic reticular activating system" because electrical stimulation of these very regions had been shown to desynchronize the cortical EEG (Moruzzi and Magoun, 1949). This idea fit well with the observation that reticular stimulation causes a large increase in acetylcholine (ACh) release from the cerebral cortex (Szerb, 1967).

With the subsequent elucidation of the monoaminergic pathways of the forebrain (Lindvall and Björklund, 1974) came the realization that AChE was often associated with catecholaminergic neurons and therefore was not a specific marker for cholinergic cells or fibers (Butcher, 1977). Thus it became apparent that strategies other than AChE histochemistry were required to identify the sources of neocortical acetylcholine. However, while not all AChE-positive cells are cholinergic, all cholinergic cells should be AChE-positive (Fibiger, 1982). Johnston et al. (1981) used this line of reasoning to identify the source of the cholinergic projections to neocortex in the rat. These investigators made excitotoxin lesions of the AChE-positive cell groups that are known to project to cortex, and measured subsequent changes in cortical choline acetyltransferase (ChAT) activity and ACh levels. They reported a 70% drop in ChAT activity after lesions of the basal forebrain. In contrast, lesions in the thalamus or brainstem produced no significant drop in the levels of this cholinergic marker. These results indicate that the extrinsic cholinergic

innervation of rat neocortex arises from cells in the basal telencephalon. In corroboration, Saper (1984) has recently shown with immunocytochemistry that of all the cells in the rat forebrain shown to project to cortex, only those in the basal telencephalon also contain ChAT. Importantly, Johnston et al. found that lesions which depleted ChAT also decreased AChE staining in cortex. The fact that the AChE staining in cortex was only affected by lesions of known cholinergic cells, and that the changes in AChE closely paralleled changes in ChAT, stands as strong evidence that AChE in the mature neocortex is a useful cholinergic marker. These findings largely vindicate Shute and Lewis and generally support the concept of an ascending cholinergic activating system.

A renewed interest in cortical cholinergic pathways has been generated by the recent demonstrations that acetylcholine is severely depleted in the cortices of individuals with Alzheimer's dementia (See Terry and Davies, 1980, for review). Attempts have been made to attribute the functional deficits associated with Alzheimer's disease, such as memory impairment and deterioration of cognitive function, to the selective loss of cholinergic inputs to the cerebral cortex. However, this conclusion is clearly premature; the contribution of cholinergic circuitry to the function of neocortex remains poorly understood. One system which promises to be of particular value in this inquiry is the striate cortex, area 17, of the cat. Since the pioneering experiments of Hubel and Wiesel (1962), the physiology of cat visual cortex has been exhaustively researched, perhaps more so than any other cortical area in any other species. Furthermore, reproducible physiological and anatomical changes in striate cortex can be induced by simple environmental deprivation paradigms, such as monocular lid closure, during a critical period that extends from approximately 3 weeks to 3 months of age (Hubel and Wiesel, 1970). Because the resultant changes in cortex accurately reflect the experience of the kitten, the suggestion has been made that they are the neural substrate of a form of learning and memory (Spinelli and Jensen, 1979).

As a first step toward better understanding the role of acetylcholine in cortex, we have examined the source and distribution of fibers in cat area 17 that are labelled by acetylcholinesterase histochemistry. We find that most of the AChE-containing fibers in striate cortex derive from the large, intensely AChE-positive neurons in the basal telencephalon. The fact that similar cells have been shown to contain ChAT in a number of species leads us to postulate that the AChE-positive fibers in cortex are in fact cholinergic. Our results show that the cholinergic input is in a strategic position to modulate visual information processing in striate cortex. A preliminary account of these data has appeared (Carnes et al., 1984).

## MATERIALS AND METHODS

### *Animals*

In all, 18 cats were used in this study: 15 mature adults over one-year of age (designated with a prefix of "C") and 3 young adults, aged between 3 months and one year (designated with a prefix of "K").

### *Surgical procedures*

All surgery was performed under aseptic conditions with intravenous (i.v.) sodium pentobarbital anesthesia. Four types of operation were caried out: Injection of horseradish peroxidase (HRP) into cortex, lesions of subcortical structures with kainic acid, surgical disconnection of a large slab of striate cortex, and surgical lesions of the midbrain. In the HRP experiment, four 0.2 µl injections of 30% HRP in phosphate buffered saline, pH 7.4, were made in the crown of the postlateral gyrus using a 1.0 µl Hamilton microsyringe. The animal survived for 48 hours at which time it was perfused for HRP histochemistry.

To produce chemical lesions in subcortical forebrain structures, a 1.0 µl Hamilton syringe was lowered to the appropriate stereotaxic coordinates, and 0.5-1.0 µl of 5 µg/µl kainate (Sigma) in saline (pH 7.6) was injected. These animals survived for 5-7 days before being perfused for AChE histochemistry.

Surgical isolation of a large slab of striate cortex was achieved using the method of subpial aspiration. First, the medial wall of a 15 mm length of suprasylvian gyrus was removed. This lesion was continued ventro-medially to the pia-arachnoid located in the

depth of the splenial sulcus. Finally, the lateral gyrus was transected anteriorly and posteriorly by aspiration of a narrow trench of cortex to leave an isolated segment of lateral gyrus with an intact blood supply. This animal survived for 2 weeks before being sacrificed.

Subpial aspiration was also used to produce a lesion in the midbrain. In this case, one hemisphere was gently retracted, and the splenium of corpus callosum removed by aspiration to expose the roof of the midbrain at the level of the superior colliculus. The midbrain was partially transected at this level on one side. The animal survived for 9 days before being perfused for AChE histochemistry.

***Eyeball injections***

In some cases, the geniculo-cortical projection fibers from one eye were labelled for comparison with the AChE pattern. This was achieved by making intraocular injections, in lightly anesthetized cats, of 1.0 mg WGA-HRP (Sigma) in 50 µl of phosphate buffered saline (Itaya & Van Hoesen, 1982). Dense transneuronal label was observed in striate cortex 48 hours after the injection.

***Tissue preparation***

All animals were deeply anesthetized with sodium pentobarbital and perfused through the ascending aorta first with 200 cc saline, then with the appropriate fixative. For HRP experiments, this fixative consisted of 2 liters of 1.25% glutaraldehyde and 1.0% formalin in phosphate buffer, pH 7.4. For AChE experiments, the fixative consisted of 2 liters of 10% phosphate buffered formalin, pH 7.4. In either case, the first liter of fixative was perfused rapidly at a pressure of 120 mm Hg, followed by a second liter perfused slowly over 20-30 minutes. This was followed by a 30 minute vascular rinse with 2 liters of cold

10% sucrose in phosphate buffer, pH 7.4. The brains were removed from the skull, blocked in the appropriate plane of section, and frozen by immersion in 2-methylbutane at -50°C for 5 minutes.

The brains were sectioned in a cryostat at 40 μm, in either the coronal or approximate horizontal plane. Adjacent sections, kept in serial order, were collected in 0.1 M phosphate buffer, pH 7.4, for Nissl staining, AChE histochemistry, and, when appropriate, HRP histochemistry.

***Histochemistry***

Two procedures were used to localize AChE. The first was a modification (Jacobowitz & Creed, 1983) of Koelle's method (Koelle, 1955). The tissue was first pre-incubated for 30 minutes at 38°C in 1.25 x $10^{-3}$ mM Iso-OMPA (a butyrylcholinesterase inhibitor) in 24% sodium sulfate. This was followed by incubation for 2-3 hours at 38°C in a solution containing 215.6 mg acetylthiocholine iodide, 2 mM copper glycine, 50 mM maleate, 2 mM copper sulfate, 80 mM magnesium chloride and 9.36 x $10^{-4}$ mM Iso-OMPA in a final volume of 187.5 ml of 24% sodium sulfate, pH 6.0. After washes in 20% and 10% sodium sulfate, the stain was developed at room temperature in 4% phosphate buffered ammonium sulfide, pH 6.0, for one minute. Next, the tissue was washed in distilled water, and the stain fixed in 10% phosphate buffered formalin, pH 7.4, for 20 minutes. The sections were again washed in $dH_2O$ and the stain toned in 0.2% gold chloride for 5 minutes. After another water wash, the toner was developed in 5% sodium thiosulfate for 5 minutes. Finally, the tissue was washed in water, mounted onto glass slides, dehydrated through a series of alcohols, cleared in xylene and coverslipped.

The second method to localize AChE was a modification (Hardy et al., 1976) of the Karnovsky and Roots method (Karnovsky & Roots, 1964). According to this method, the

sections were incubated for approximately 18 hours in a medium containing 226 mg acetylthiocholine iodide, 12.5 mg promethazine (a butyrylcholinesterase inhibitor), 10 mM glycine and 2 mM copper sulfate in a final volume of 200 ml of 0.05 M acetate buffer, pH 5.0, at 38°C. Next, the sections were thoroughly washed in saline and reacted for one minute in 1.25% sodium sulfite. After a water wash, the stain was developed in 1% silver nitrate for 5 minutes. The stain was then fixed in 5% sodium thiosulfate for 3 minutes. After another saline wash, the sections were mounted onto slides, dehydrated, cleared and coverslipped.

The AChE reaction product was examined with a Leitz Orthoplan microscope using normal brightfield and interference contrast optics. Both staining methods yielded essentially the same pattern of staining in striate cortex. However, individual AChE-positive fibers were only seen with the modified Koelle method. The modified Karnovsky and Roots method was only used because of its simplicity and because it amplified the stain for low-power microscopy. All the stain in striate cortex was abolished by the addition of $10^{-5}$ M eserine sulfate to the substrate incubation medium, regardless of the method.

Sections were reacted for HRP histochemistry according to Mesulam's (1978) procedure. The tissue was first pre-incubated in a solution containing 100 mg sodium nitroferricyanide and 5 mg tetramethylbenzidine (TMB) in 100 ml of 10 mM acetate buffer, pH 3.3. The reaction was started by adding 5 ml of 0.3% $H_2O_2$ to this solution. The tissue was reacted in several changes of this medium for 20 minutes. After six washes in cold 0.1 M acetate buffer, pH 3.3, the sections were mounted onto glass slides. The tissue was then dehydrated in a dessicator overnight at 5°C at which time the TMB reaction product was stabilized in methyl salicylate (Adams, 1980) and the tissue cleared in xylene and coverslipped. This material was examined on a Leitz Orthoplan microscope using crossed-polarized optics.

## RESULTS

### *The pattern of acetylcholinesterase staining in striate cortex*

The normal organization of cholinesterase-positive fibers in cat striate cortex, as revealed by the modified Koelle method, is illustrated in figure 1. This feltwork of profusely branched fibers is clearly stratified into several plexuses of tangentially-running axons. The most prominent plexus occurs in layer I (figure 3). In addition, two plexuses of stained axons occur in the middle cortical layers, and another in deep layer VI. In figure 2, these fiber plexuses are related to the Nissl cytoarchitecture of an adjacent section. One of the middle bands of AChE lies just above layer IV in deep layer III; the other band actually lies within layer IVc. This was confirmed also by labelling thalamic inputs on sections adjacent to those reacted for AChE (not illustrated). We emphasize that these bands relate to the density and orientation of the AChE-positive fibers, not necessarily to the density of their synapses.

In addition to the stained fibers in striate cortex, a population of pyramidal cells in layer V contained AChE. These were observed in all of our preparations, but were particularly striking in cortex fixed with glutaraldehyde (figure 4). The reaction product is found in the cytoplasm of the perikaryon and extends only a short distance into the proximal segment of the apical dendrite. As illustrated in figure 5, the AChE-positive cells constitute only one subset of layer V pyramidal cells. Another population of less densely AChE-positive neurons, was also observed, but normally only in cases fixed with glutaraldehyde. These cells were found primarily in lower layer III in a zone that corresponded to the layer III plexus of AChE-positive fibers.

AChE-positive axons were regularly observed coursing through the white matter of the lateral gyrus. However, their appearance depended on the plane of section (figure 6). Stained axons could be traced for considerable distances in horizontal sections, while only short segments were observed in coronal sections. These observations indicate that AChE-positive axons have an anterior-posterior trajectory in the cortical white matter.

While our attention has been directed primarily toward AChE in area 17, it should be mentioned that we observed no differences in the staining pattern of area 18. However, area 19 did differ from areas 17 and 18. The main difference was the disappearance of the distinct mid-cortical AChE bands at the 18/19 border.

***Identification of possible sources of cortical AChE***

We next sought to identify the possible source(s) of the AChE-positive axons in striate cortex. Our strategy involved backfilling with HRP the cells in the forebrain that project to visual cortex, and reacting adjacent sections for AChE. Four large HRP injections were made in the crown of the postlateral gyrus, involving both areas 17 and 18, and 48 hours later the brain was processed for HRP histochemistry. The results of this experiment are illustrated in figure 7. In addition to the labelled cells in the lateral geniculate nucleus, we found HRP-positive cells in the lateral posterior nucleus (figure 7: 6.68-7.40), in the anterior intralaminar nuclei (figure 7: 8.60-9.72), and in the dorsal claustrum (figure 7: 8.60-10.44). In addition, we found labelled cells scattered within the basal telencephalon (figure 7: 10.44-12.36).

Of the cell populations in the forebrain that were retrogradely labelled with HRP, only the lateral geniculate nucleus, the intralaminar nuclei, and the basal telencephalon contained significant AChE reaction product. The former two groups were characterized by dense neuropil staining that obscured any AChE-containing cell bodies. However, many

large, intensely AChE-positive neurons were observed in the region of the basal telencephalon that contained retrogradely-labelled cells (figures 8 and 9). In figure 8A, the location of labelled cells is plotted on an adjacent Nissl-stained section through the basal forebrain. Particularly note the labelled cells that are embedded in the internal capsule. Figure 8B shows this same region stained for AChE. At higher power, in figure 9, it can be seen that many intensely-reactive neurons are also embedded in the internal capsule. In fact, counterstaining with cresyl violet shows that virtually all the neurons within the internal capsule are intensely AChE-positive.

***The effects of forebrain lesions on cortical AChE***

To examine the possible contribution of each of these subcortical structures to the AChE staining pattern in striate cortex, we made a series of subcortical lesions with kainic acid. We chose this excitotoxin because available data suggest that in the proper concentration it destroys neurons, but spares axons of passage (Coyle, 1983). In our first experiment (C-114), we injected 5 µg kainate in 1 µl of saline into the basal forebrain. From the inter-aural line, the stereotaxic coordinates of the injection were anterior 15.0 mm, lateral 5.5 mm, and dorsal 11.0 mm. This produced a large lesion, centered in the internal capsule, that destroyed cells in the surrounding caudate, putamen, globus pallidus, dorsal claustrum, and substantia innominata (figures 10 and 11). The lesion did not involve cells in the thalamus. Subsequent AChE histochemistry revealed a significant depletion of the enzyme in cortex on the lesioned side (figure 11). The AChE stain was reduced in all the cortical areas examined, including a large part of area 17 (figure 12).

A low power survey of sections from C-114 reacted according to the modified Karnovsky and Roots method indicated that AChE was virtually eliminated in some regions of area 17 (figure 11 for example). However, closer examination of adjacent sections stained with the modified Koelle method showed that a considerable number of AChE-positive fi-

bers could still be demonstrated by this technique even in the most severely-depleted regions. Nonetheless, the density of AChE-containing fibers in area 17 appeared to be reduced on the lesioned side to less than half of that on the control side (figure 13). This reduction was not restricted to any one layer, but rather seemed to involve all layers equally.

Smaller lesions of the basal telencephalon were also sufficient to deplete AChE in widespread regions of the neocortex. In C-116, for example, an injection of 2.5 μg of kainate in 0.5 μl saline produced a lesion that destroyed many neurons situated in the internal capsule but largely spared the caudate and globus pallidus (figure 14A). Reconstruction of the brain from AChE-stained sections revealed a complicated pattern of depletion in frontal and temporal areas of cortex (figure 14B). Only partial depletion of area 17 was observed in this case, and this was restricted to the cortex anterior to the representation of the horizontal meridian (Tusa et al., 1978). However, a small lesion placed more ventromedially, in C-120, was effective in depleting AChE in occipital cortex, including all of area 17 (figure 15).

The two other cell groups in the forebrain that we considered as possible candidates for providing a cholinergic input to visual cortex were the anterior region of the intralaminar nuclei (CL nucleus) and the lateral geniculate nucleus. The intralaminar nuclei were injected with kainate in C-117 (figure 16). In figure 17 we relate the location of the lesion in C-117 to the position of labelled cells after HRP injections of visual cortex (from K-211). It appears that this lesion destroyed most of the cortically-projecting neurons in the central lateral nucleus (CL) and many in the para-central nucleus (PC). Nevertheless, the cortical AChE pattern was not detectably affected by this lesion.

The lateral geniculate nucleus was injected with 4 μg of kainate in C-118. The lesion involved most of the nucleus throughout its anterior-posterior extent. No change in the

pattern or density of AChE-containing fibers was observed in striate cortex one week after this lesion. Together, these data indicate that of all the cell groups in the forebrain known to project to visual cortex, only the basal telencephalon provides an AChE-positive innervation.

***The effects of surgical undercutting on cortical AChE***

While the basal forebrain lesions always depleted AChE in striate cortex, the depletion was never complete. A significant population of AChE-positive fibers always survived (figure 13). Because cells that contained AChE were present in cortex, we wondered if the remaining fibers might be part of an intrinsic network. To examine this possibility, a length of postlateral gyrus was surgically isolated from one hemisphere in C-118. Two weeks later the animal was processed for AChE histochemistry. The isolated region appeared healthy and its blood supply was not detectably compromised. Nonetheless, there was a striking and nearly complete (> 99%) loss of AChE-positive fibers (figure 18). An occasional axon could be observed in layer I, but fibers deeper in cortex were exceedingly rare. It is likely that the remaining fibers crossed a small bridge of tissue that was missed during surgery. Despite the dramatic reduction in stained fibers, significant neuronal cell body reactivity remained (figure 19). Besides the stained layer V pyramidals, many layer III cells contained AChE reaction product, even in this formalin-fixed material (which is not optimal for cell bodies). However, none of these AChE-positive neurons were ever observed giving rise to stained fibers. In addition, close examination of the subcortical targets of layer V, such as the superior colliculus and the intralaminar thalamus, revealed no changes in their AChE content.

The intact cortex located immediately posterior to the undercut piece also displayed a marked depletion of AChE as compared to the control hemisphere or to cortex anterior to the undercut. This probably resulted from the transection of axons running in the white

matter of the lateral gyrus. That this depletion was seen posterior, but not anterior, to the lesion further indicates that the cholinesterase-positive axons travelling in the white matter are coursing from anterior to posterior in the hemisphere.

### *The effects of midbrain lesions on forebrain AChE*

Mufson et al. (1982) recently described a projection to neocortex in the Rhesus monkey that arises from intensely AChE and ChAT reactive neurons in the region of the tegmentum called "nucleus cuneiformis." While we found no HRP labelled cells in this region after injections of visual cortex in the cat, we nevertheless felt compelled to explore the possible contribution of cells in the tegmentum to the cortical AChE pattern. Thus, in C-121, we made a large surgical lesion in the tegmentum at the level of the superior colliculus (figure 20). We found that several nuclei of the dorsal thalamus were partially depleted of AChE by the lesion. The most conspicuous changes were seen in the lateral geniculate nucleus (figure 20B) and the lateral intermediate nucleus (figure 20C) on the lesioned side. However, this lesion produced no detectable decrease in AChE-positive fibers in visual cortex.

## DISCUSSION

### *The pattern of cholinesterase staining in striate cortex*

The pattern of AChE staining we observed in striate cortex generally agrees with the description of Krnjevic and Silver (1965) for cat neocortex. In that study, using the original Koelle method they showed that cortex received an extrinsic AChE-positive innervation that formed a deep network in layer VI, and also a superficial plexus in layer I. Layers IV and V contained a zone of diffuse staining that was not clearly associated with fibers. Layers II and III were least stained. These generalizations seem to apply to striate cortex as well. However, in addition to the particularly rich plexus in layer I, we observed two middle plexuses of stained axons. One clearly lies just above layer IV, among the pyramidal cells of deep layer III. The other is at the layer IV-V border, lying mostly in layer IVc. Layers II, upper III and IVa+b had the fewest stained fibers. Nonetheless, significant numbers of axons traversed these layers en route to layer I.

It is interesting to note that while the basic pattern of AChE stain is quite robust, the details are dependent on the histochemical method and the tissue processing. For example, while individual fibers were readily discerned with the modified Koelle method, the modified Karnovsky and Roots method yielded only a diffuse stain. Thus, while both methods showed the laminar distribution of the enzyme, only the Koelle method clearly delineates the AChE contained within axons. Moreover, the appearance of the stain varied considerably with the type of fixative. With formalin fixation, virtually all of the stain in cortex was restricted to axons. Only layer V neurons had any reaction product. In contrast, many reactive neurons outside of layer V were apparent in tissue fixed with glutaraldeh-

yde. The trade-off was a much lower quality fiber stain. The different fixatives probably are biased toward preserving either the soluble or membrane-bound forms of the enzyme; glutaraldehyde favoring the soluble fraction, formalin favoring the membrane-bound fraction.

Regardless of the method, a consistent observation was the existence of a population of reactive neurons in layer V. However, not all cells stained, but rather a subpopulation of pyramidal cells that were among the largest in the layer. The staining appeared to be cytoplasmic and extended only short distances into the proximal, apical dendrite. Stained axons could not be observed in continuity with these cells. The distribution and size of the stained cells is reminiscent of those Gilbert and Kelly (1975) showed to project to the superior colliculus. It is possible that these pyramidal cells form a distinct class of striate cortical neurons that share similar histochemistry and connectivity. This possibility awaits examination using double-label methods.

Cortical cholinesterase staining appears to be a feature shared by all mammals and available data indicate that the pattern is also quite conservative, with layer I being most densely stained. However, significant cytoarchitectural variations do appear; for example, the abrupt alteration in the AChE pattern at the area 18-19 border. Similar changes have been noted at the 17-18 border in *Galago senegalensis* and *Aotus trivirgatus* (Fitzpatrick and Diamond, 1980). In addition, it is also clear that significant differences in the stain occur between species. For example, Fitzpatrick and Diamond (1980) report that AChE distributes mainly in layers I, IV and VI in the striate cortex of *Galago* and *Aotus*. The pattern in cat differs from this arrangement, with most of layer IV being devoid of stain and layer III possessing a prominent plexus of stained axons. In addition, Fitzpatrick and Diamond found a population of intensely reactive neurons in layer VI. Likewise, Krnjevic and Silver found AChE-positive layer VI cells in some areas of cat cortex, such as the suprasylvian gyrus. In contrast, we found no evidence for AChE reactive layer VI cells in

cat area 17. Together, these observations indicate that caution should be exercised in generalizing about cortical cholinesterase patterns across neocortical subdivisions or across species.

### *The source of AChE in visual cortex*

Cell groups in the forebrain were considered to be possible candidates for providing a cholinesterase-positive innervation of striate cortex if they fulfilled two criteria: 1) retrograde label after visual cortical HRP injections and 2) intense AChE staining. Of the cell groups labelled with HRP, only the LGN, the anterior intralaminar nuclei and the basal telencephalic nuclei contained significant AChE. However, only lesions of the basal forebrain depleted cortical AChE; destruction of the lateral geniculate nucleus or the intralaminar nuclei did not result in any detectable loss of AChE in striate cortex. This result was certainly expected for the LGN since pharmacological studies had indicated that ACh was not the synaptic transmitter of specific visual afferents to striate cortex (Krnjevic and Phillis, 1963a, 1963b; Spehlmann et al., 1971). In contrast, a number of physiological and pharmacological studies have indicated that the projection of the intralaminar nuclei upon cortex might be cholinergic. For example, stimulation of the intralaminar thalamus desynchronizes the cortical EEG (Moruzzi and Magoun, 1949), an effect that can be partially blocked with the muscarinic antagonist atropine (Bradley and Elkes, 1957). Atropine also blocks the unit responses of many neurons to intralaminar stimulation. Nonetheless, our results indicate that the intralaminar nuclei do not themselves provide cholinesterase-positive fibers to visual cortex. In fact, the very existence of afferents to striate cortex from these nuclei has recently been challenged by Raczkowski and Rosenquist (1983). They find that only HRP injections involving area 18 backfill cells in CL. Injections that are unequivocally restricted to area 17 do not label cells in these intralaminar nuclei. Our large in-

jections certainly involved the area 17-18 border zone as evidenced by the trans-callosal label. This apparent paradox is resolved by the finding that 1) the intralaminar nuclei project heavily into the basal telencephalon (Marshall and Beckstead, 1983), and 2) lesions of the basal telencephalon significantly deplete AChE in visual cortex. Thus, the intralaminar nuclei may transmit information to striate cortex via a synaptic relay with cholinergic neurons in the basal forebrain.

That the basal telencephalon provides AChE-positive innervation of cat neocortex is not remarkable in light of similar reports in rodents (Johnston et al., 1981; Wenk et al., 1980). In fact, Krnjevic and Silver (1965) originally identified this possibility in cats based on their observation of fibers in cortex that were continuous with cells in the basal forebrain. However, the distribution of cells in the basal forebrain that project to visual cortex is surprisingly widespread. Backfilled cells after HRP injections of visual cortex were not clustered in this region, but rather were widely scattered among cells in the ventromedial globus pallidus, the substantia innominata, the diagonal band of Broca and embedded among the fibers in the internal capsule. A similar distribution has been reported by Tigges and Tigges (1984). While these neurons are not easily classified according to their position in the forebrain, they may be operationally defined according to the Mesulam's criteria as Ch-4 neurons (Mesulam et al., 1983). Ch-4 cells are the AChE-rich neurons of the basal telencephalon that project to neocortex.[4] Because of this widespread distribution of Ch-4 neurons in the cat, even large kainate lesions are likely to spare many cells that project to visual cortex. This may account for the only partial depletion of AChE we observed in striate cortex after lesions of the basal forebrain.

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[4] The one exception is the AChE-positive cells in the diagonal band of Broca which Mesulam designated as Ch-3. For simplicity, we will refer to all the AChE-positive cells in the basal forebrain that project to visual cortex as Ch-4 neurons.

On the other hand, another possible source of remaining fibers after basal forebrain lesions is the intensely AChE-positive cells in layer V. Indeed, these cells remained reactive even two weeks after complete surgical isolation of striate cortex. However, the fact that undercutting eliminated virtually all the stained axons in cortex, suggests that the layer V cells do not contribute to the normal pattern of stained fibers. A second possible source of cortical AChE is the brainstem since Mufson et al. (1982) found intensely AChE-positive cells (that also contain ChAT) in the Rhesus monkey "nucleus cuneiformis" that project to cortex. To examine this possibility, a large lesion of the midbrain was made at the level of the rostral superior colliculus. While this lesion caused some depletion of AChE in the thalamus, we could not detect any loss of AChE in cortex. Finally, we dismissed the possibility that any AChE-positive fibers arose from the noradrenergic, but nonetheless AChE-reactive neurons in the locus coeruleus based on two observations. First, the laminar distribution of noradrenergic fibers in striate cortex is significantly different from the AChE pattern we observed (Itakura et al., 1981). Second, depletion of norepinephrine with neonatal injections of 6-hydroxydopamine has no effect on AChE in cortex (unpublished observation).

Together, these data suggest that the large intensely AChE-positive neurons of the basal telencephalon are the sole source of AChE-positive axons in cat striate cortex. It is not possible to make any definite statements about the topography of the projection based on our results. However, two points seem relevant. First, the AChE depletion caused by lesions of the basal forebrain did not seem to bear any simple relationship with cytoarchitectural borders. Second, while a single lesion of the basal forebrain was sufficient to deplete AChE in widespread areas of cortex, it was not sufficient to deplete completely any single cortical area. These observations suggest a number of conclusions. First, Ch-4 cells projecting to different cortical areas must be intermingled. Second, Ch-4 cells projecting to a given area must be widely dispersed. Third, individual Ch-4 cells that project

to a given area must have widespread arborizations. A number of investigators have recently employed double-label techniques to investigate the terminal arbors of Ch-4 axons in rat neocortex (Price and Stern, 1983; Saper, 1984). The general conclusion is that most Ch-4 cells project only to a single cortical area. However, this does not exclude the possibility of large degrees of overlap *within* a cortical area (such as striate cortex). The cat might be the ideal species to resolve these questions. Virtually all the neurons in the internal capsule are AChE-positive and most of these cells, at least at the level of the anterior commissure, project to the neocortex. It appears then that these large neurons would be accessible to intracellular injections of HRP. It is possible with this method to label an axonal arbor in its entirety (Kitai et al., 1976; Gilbert and Wiesel, 1979).

***Are AChE-positive elements cholinergic?***

A number of lines of evidence suggest that the AChE-positive axons we observe in striate cortex are in fact cholinergic. First, the development of AChE and ChAT in cat striate cortex are closely correlated (Potempska et al., 1979). Second, the distribution of AChE-stained axons agrees with the distribution of muscarinic receptor sites as shown using autoradiography (Shaw et al., 1984). Third, non-cholinergic inputs whose cells express AChE, such as the lateral geniculate nucleus and the locus coeruleus, do not contribute AChE-positive axons to striate cortex. Finally, the strongest evidence is that AChE-positive axons in striate cortex arise from Ch-4 neurons in the basal telencephalon. These intensely AChE-positive cells have been shown by immunocytochemistry to contain choline acetyltransferase in a number of species. Furthermore, lesions of these cells cause a parallel drop in the cortical level of ChAT and AChE (Johnston et al., 1981). It therefore appears likely that the cholinesterase-positive axons we describe in striate cortex release acetylcholine at their endings.

The situation is less clear for the AChE-positive neurons in striate cortex. On one hand, Hebb et al. (1963) found that about 10% of the ChAT activity remained in cat neocortex after undercutting. Our results indicate that the only significant AChE that remains in cortex after surgical isolation is associated with neurons, particularly those in layer V. Together, these observations suggest that some fraction of layer V neurons in area 17 might be cholinergic. On the other hand, the subcortical targets of layer V did not display any reduction in AChE after cortical isolation. Furthermore, close examination of the AChE-positive cells, in either normal material or after undercutting, did not reveal any stained axons leaving these neurons. The few axons we did observe in the undercut cortex appeared in layer I and probably crossed a small bridge of intact tissue. It seems plausible that the remaining ChAT activity seen by Hebb et al. similarly might have been associated with axons that were missed during surgery. Clearly, the final resolution of this question will require ChAT immunocytochemistry.

If the layer V cells are not in fact cholinergic, then it is natural to question why they contain so much AChE. Perhaps these cells synthesize this degradative enzyme because they are a prime target of cholinergic afferents. Indeed, laminar analysis of cholinoceptive units in cat visual cortex reveals that the vast majority lie in layer V (Krnjevic and Phillis, 1963a).

### *Functional considerations*

Acetylcholine historically has been regarded as the cortical transmitter of the ascending reticular activating system. A large body of evidence is consistent with this suggestion (see Singer 1979, for review). This activating system may be defined as those subcortical structures that, when electrically stimulated, desynchronize the cortical EEG (Moruzzi and Magoun, 1949). These structures include the midbrain reticular formation (MRF) and the midline and intralaminar thalamic nuclei. With the discovery that the intralaminar nuclei

project directly to widespread regions of cortex, came the suggestion that this was the final common pathway for cortical activation. The present results indicate, however, that any cholinergic component of cortical activation by thalamic stimulation must involve a relay with the cholinergic cells of the basal telencephalon. In this context, it is interesting to note that while atropine blocks the desynchronization of the EEG, it apparently has no effect on the recruiting response, although both may be elicited by stimulation of the intralaminar thalamus (Loeb et al., 1960). It thus appears likely that reticular activation of cortex involves both direct thalamo-cortical mechanisms and indirect pathways that relay with cholinergic cells in the basal telencephalon. Consistent with this idea is Spehlmann's (1971) observation that both cholinoceptive and non-cholinoceptive cortical units are activated by MRF stimulation. Accordingly, only the activation of cholinoceptive units was blocked by iontophoretic atropine. Moreover, Jasper and Koyama (1969) found that stimulation of different parts of the reticular activating system selectively stimulated the release of either ACh or glutamate from the cortical surface.

The exact role of acetylcholine in the function of cerebral cortex must remain the subject of speculation. About 20% of cortical neurons are depolarized by ACh. This effect is antagonized by the muscarinic antagonist atropine. Muscarinic activation is thought to stimulate the production of cyclic nucleotides in the target cells. Thus, the metabolic effects of ACh on a cortical neuron may far outlast any transient changes in membrane conductance. Cholinergic enzymes, especially AChE, show provocative changes in kitten striate cortex during the critical period when area 17 is modifiable by the visual environment (Potempska et al., 1979). It is tempting to speculate that ACh may act as a modulator of neuronal plasticity during this period. Indeed, Singer (1982) has found that lesions of the intralaminar thalamus will abolish the normal response of kitten striate cortex to extended periods of monocular deprivation. Since the intralaminar nuclei may not even project to area 17, it is possible that the lack of cortical plasticity seen in these kittens is a consequence of indirectly silencing the cholinergic projection.

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## Appendix A

## ABBREVIATIONS AND FIGURE LEGENDS

### ABBREVIATIONS

#### *Thalamic Nuclei*

| | | | |
|---|---|---|---|
| AM | Anterior Medial | PC | Para-Central |
| AV | Anterior Ventral | Pul | Pulvinar |
| CL | Central Lateral | Re | Reticularis |
| HA | Anterior Hypothalamus | Sub | Subthalamus |
| LD | Lateral Dorsal | VA | Ventral Anterior |
| LGN | Lateral Geniculate | VB | Ventral Basal |
| LI | Lateral Intermediate | VL | Ventral Lateral |
| LP | Lateral Posterior | VM | Ventral Medial |
| MD | Medial Dorsal | ZI | Zona Inserta |

#### Other

| | | | |
|---|---|---|---|
| AC | Anterior Commissure | F | Fornix |
| Am | Amygdala | GP | Globus Pallidus |
| Ca | Caudate | Ha | Habenula |
| Cld | Dorsal Claustrum | IC | Internal Capsule |
| Clv | Ventral Claustrum | LV | Lateral Ventricle |
| CP | Cerebral Peduncle | NS | Septal Nuclei |
| DBH | Diagonal Band of Broca, Horizontal Limb | OT | Optic Tract |
| | | Put | Putamen |
| DBV | Diagonal Band of Broca, Vertical Limb | SI | Substantia Innominata |
| | | III | Third Ventricle |

### FIGURE LEGENDS

FIGURE 1: Cat striate cortex reacted for AChE according to the modified Koelle method. Pia is at the top, white matter at the bottom. In this case the tissue was also lightly counterstained with eosin.

FIGURE 2: The Nissl architecture of striate cortex (A) related to an adjacent section reacted for AChE histochemistry (B). The superficial plexus of AChE-positive fibers lies entirely within layer I; the deep plexus of AChE-positive fibers lies in lower layer VI; the two middle plexuses lie in lower layer III and in layer IVc, respectively.

FIGURE 3: A high-power photomicrograph of the AChE-positive axons in layer I.

FIGURE 4: AChE-positive pyramidal cells in layer V. These cells were seen in all cases, but were particularly conspicuous in tissue fixed with glutaraldehyde. In this case, the tissue was also lightly counterstained with eosin.

FIGURE 5: A camera lucida drawing of layer V neurons. Filled profiles represent AChE-positive cells, open profiles represent Nissl-stained cells that lack AChE reaction product. Note that the AChE-positive pyramidals are among the largest cells in layer V.

FIGURE 6: AChE-stained axons in the white matter of the postlateral gyrus. A: Profiles seen in horizontal sections. B: Profiles seen in coronal sections. This difference indicates that AChE-positive axons have an anterior-posterior trajectory in the white matter. Scale bar = 50 μm.

FIGURE 7: The location of retrogradely-labelled cells, 48 hours after 4 large HRP injections in the lateral gyrus, is plotted on a series of coronal sections. The approximate level of each section is indicated on the dorsal reconstruction (upper left). The injection centers are marked with white stars; the regions of HRP uptake are shaded black. The injections

involved both areas 17 and 18. In the *posterior diencephalon* backfilled cells were found in the LGN and LP (6.68-7.40). In the *anterior diencephalon* backfilled cells were found in PC and CL (8.60-9.72). In the *basal telencephalon*, labelled cells were found in the dorsal claustrum and also scattered within the internal capsule, the ventromedial globus pallidus, the substantia innominata, and the diagonal band of Broca (10.44-12.36).

FIGURE 8: A: Cells labelled with HRP after visual cortical injections are plotted onto an adjacent Nissl-stained section through the basal telencephalon (from K-211). Each dot represents a single HRP-filled neuron. B: An adjacent section reacted for AChE.

FIGURE 9: Large AChE-positive neurons within the internal capsule. A: Low power photomicrograph of the cat forebrain reacted for AChE. Some of the large, darkly stained neurons in the internal capsule are indicated by arrows. The boxed region is enlarged in B. B: High power photomicrograph of an AChE-reactive neuron embedded in the internal capsule.

FIGURE 10: Reconstruction of the kainate lesion in C-114. In this case, 5 µg of kainic acid were injected into the basal forebrain at the level of the anterior commissure. The regions of cell loss are indicated by cross-hatching. The approximate level of each coronal section is indicated on the dorsal reconstruction (upper left). A photomacrograph of section 14.44 appears in figure 11A.

FIGURE 11: A: Photomacrograph of a Nissl stained section through the telencephalon of animal C-114. The outline of the kainate lesion is indicated by a dashed line. The approximate level of this section (14.44) is indicated on the reconstruction in figure 12. B:

Photomacrograph of a section through visual cortex reacted for AChE by the modified Karnovsky and Roots method. The level of this section is shown in figure 12. Note the severe depletion of AChE in the cortex on the lesioned side, including area 17. Filled arrows indicate regions we interpret as having less than half the stain of the control side. Open arrows indicate regions that are depleted of AChE, but not by more than 50%. The topography of cortical AChE depletion caused by this lesion is illustrated in figure 12.

FIGURE 12: Surface reconstruction of the cortex of C-114 to show the extent of AChE depletion caused by the basal forebrain lesion illustrated in figures 10 and 11A. Blackened regions correspond to zones we interpret to be depleted by more than 50%; cross-hatched regions correspond to zones that are depleted of AChE, but by less than 50%. These criteria were illustrated in figure 11B. Cortex anterior and posterior to the shaded areas was not analyzed.

FIGURE 13: A comparison of the striate cortex in C-114 on the control (A) and lesioned (B) sides stained for AChE by the modified Koelle method. Note that while AChE-reactive axons are significantly reduced in all layers, a considerable number of fibers remain. Also conspicuous are the AChE-positive pyramidal cells in layer V (arrow heads).

FIGURE 14: A: The kainate lesion in C-116 is indicated by cross-hatching on a coronal section through the forebrain. B: A reconstruction of the cortical AChE depletion caused by the lesion shown in A. Conventions are the same as in figure 12. A complicated pattern of AChE depletion is apparent in frontal and temporal areas of cortex. AChE in occipital cortex, however, was largely spared by this lesion.

FIGURE 15: A: The kainate lesion in C-120 is indicated by cross-hatching on a coronal section through the forebrain. B: A reconstruction of the cortical AChE depletion caused by the lesion shown in A. Conventions are the same as for figure 12. This lesion severely depleted AChE in virtually all of striate cortex.

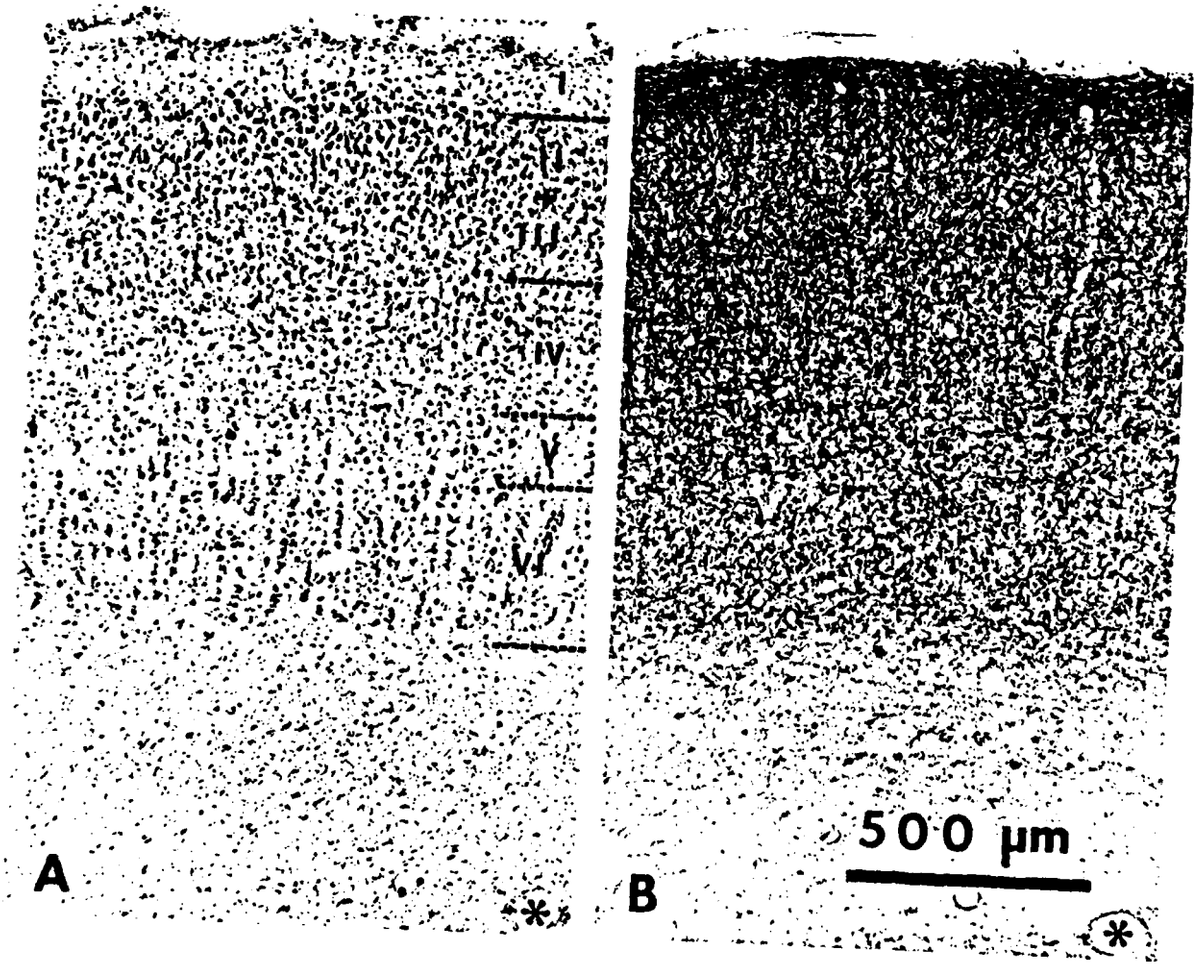
FIGURE 16: Low power photomicrograph of a Nissl-stained section through the anterior intralaminar thalamus in C-117. In this case, one side was injected with kainic acid; the lesion is indicated by a dashed line. The star labels a region of gliosis near the cannula track. This lesion caused no detectable depletion of AChE in cortex.

FIGURE 17: A: The location of cells in the intralaminar thalamus that are labelled after visual cortical injections of HRP (from K-211). B: The extent of the kainate lesion in C-117. It appears that the lesion destroyed most of the cortically-projecting neurons in CL.

FIGURE 18: The AChE stain in control striate cortex (A) is compared to that in surgically isolated striate cortex (B). There is a virtually complete loss of AChE-positive fibers in the undercut cortex. C: Striate cortex just posterior to the isolated region stained for AChE. This region is also depleted of AChE, presumably because of the transection of axons travelling in the white matter of the lateral gyrus.

FIGURE 19: The isolated striate cortex as it appears stained for AChE by the modified Karnovsky and Roots method. Particularly note the reactive cells in layers V and III.

FIGURE 20: A: Photomacrograph of a Nissl-stained section through the rostral midbrain in C-121. Note the extent of the tegmental lesion on the left side. B: An AChE-stained section through the thalamus of the same animal. The lateral geniculate nucleus of the lesioned side (filled arrow) has significantly less AChE than the nucleus on the control side (open arrow). C: A section through the rostral thalamus stained for AChE. The lateral intermediate nucleus on the lesioned side (filled arrow) is severely depleted of AChE. Despite the thalamic depletion caused by this lesion, AChE in cortex was not detectably affected.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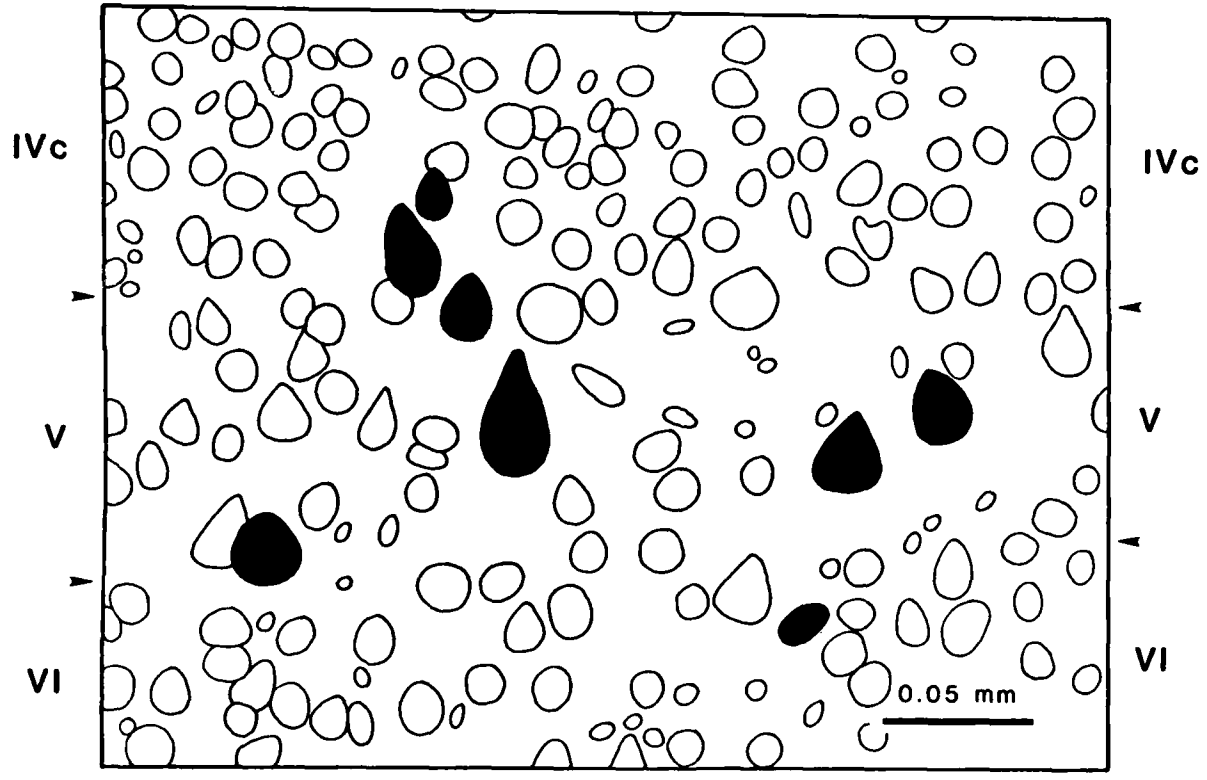
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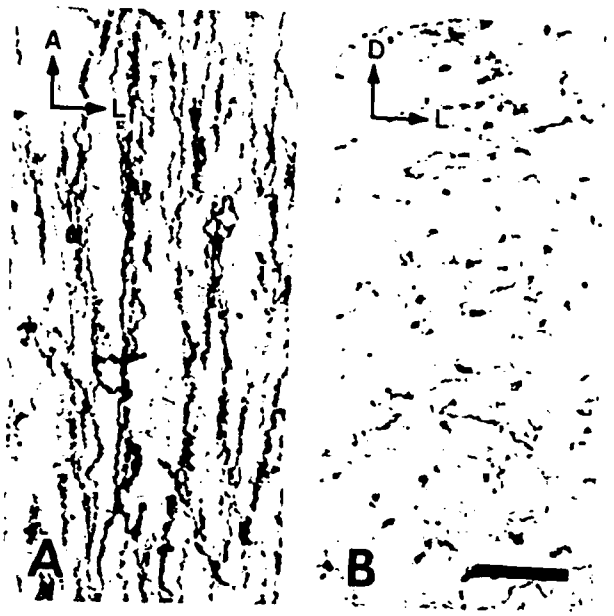
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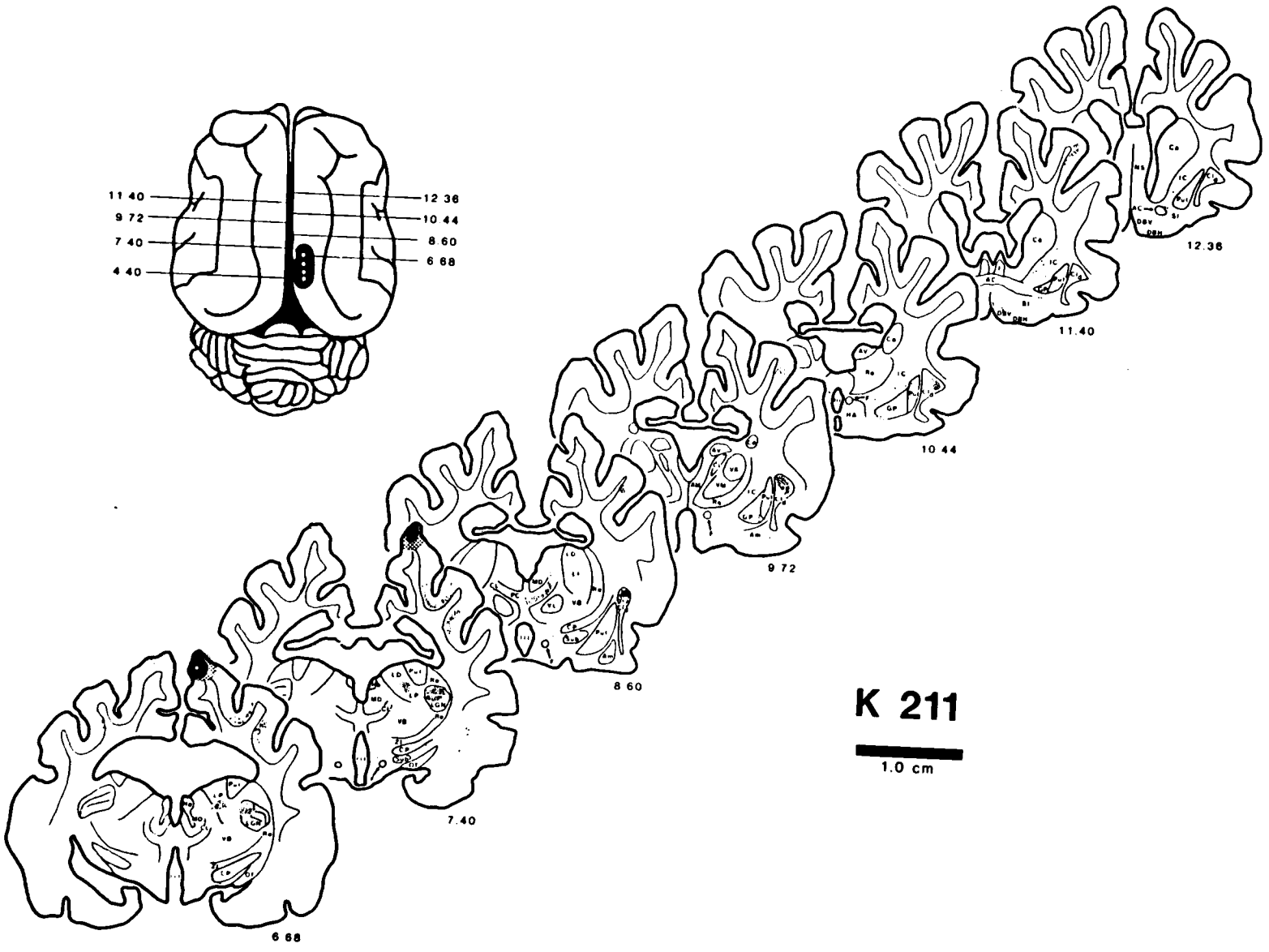
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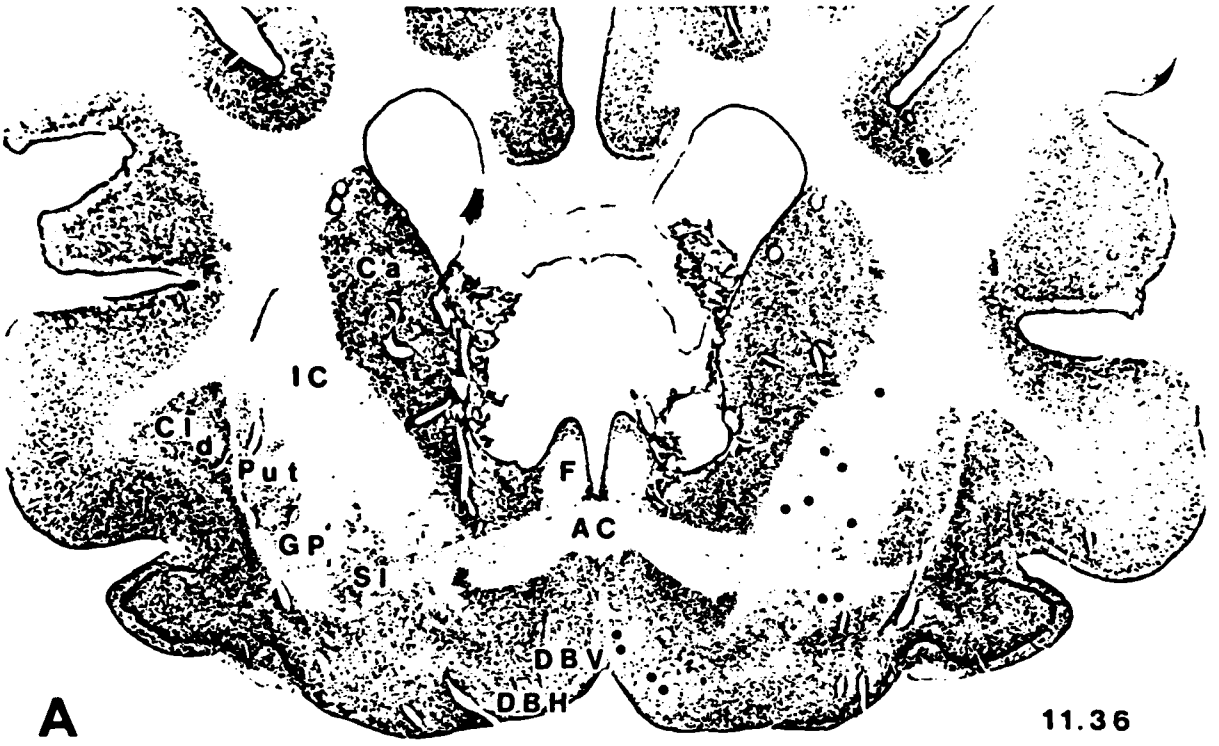
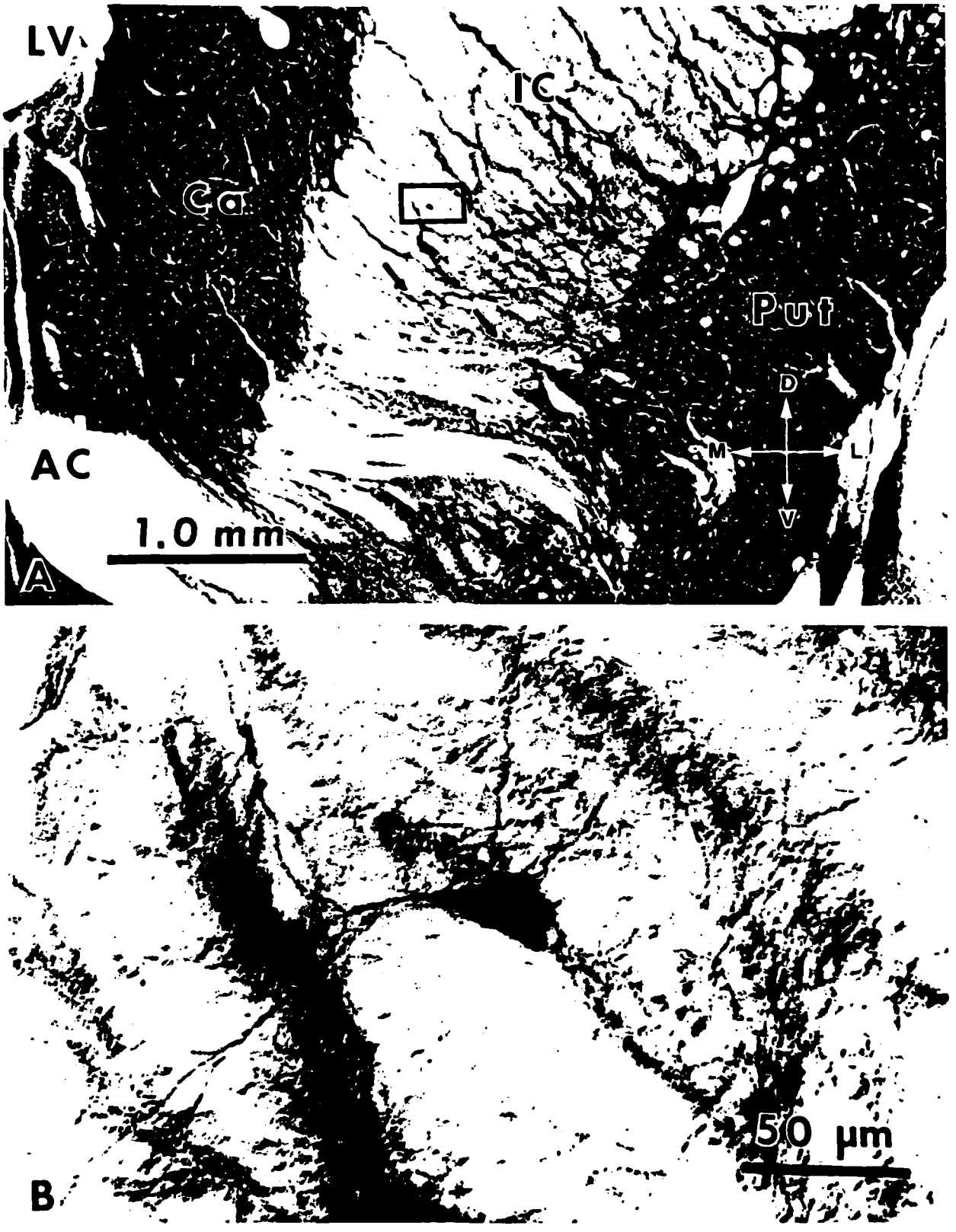


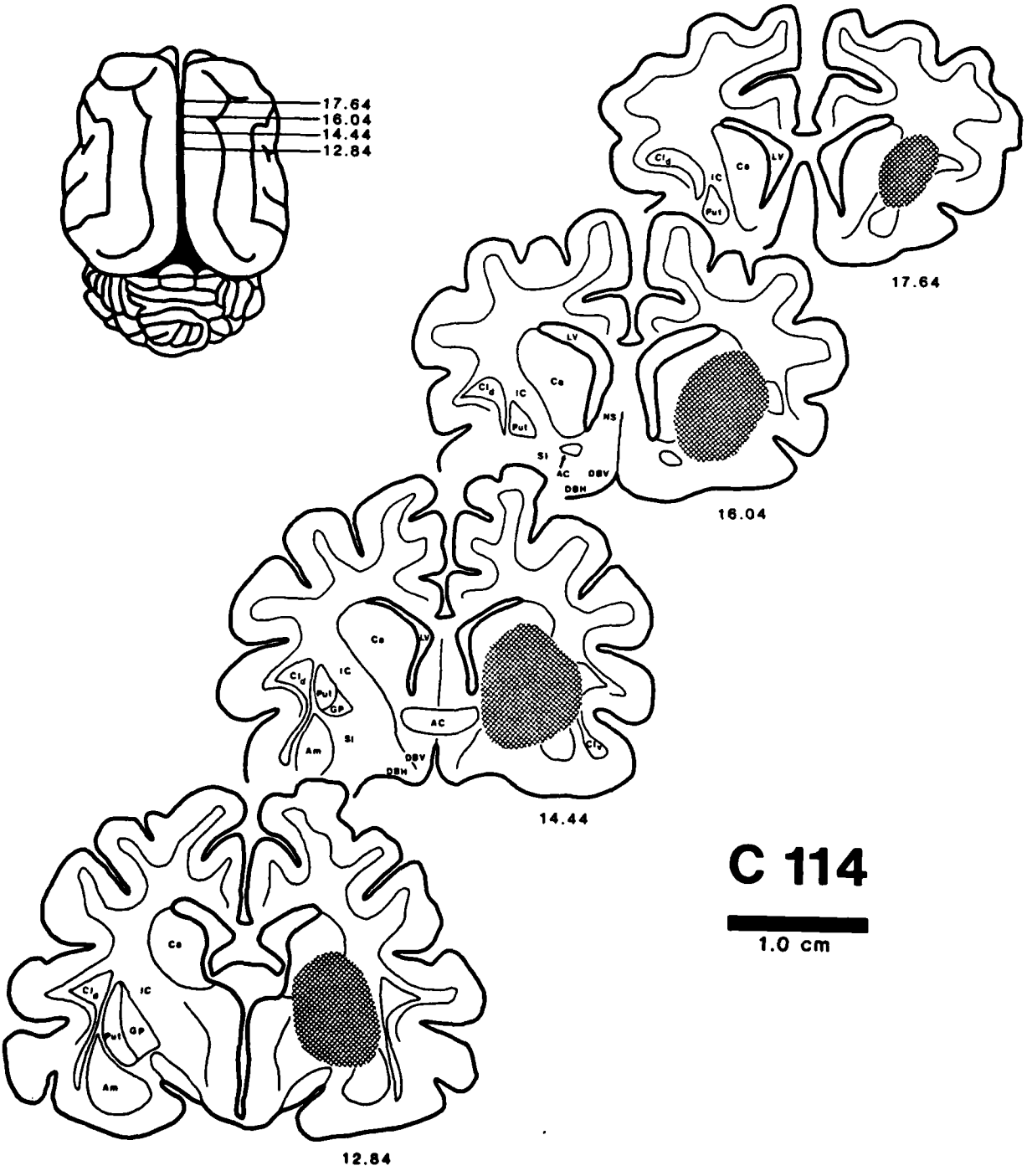

A

11.36

B

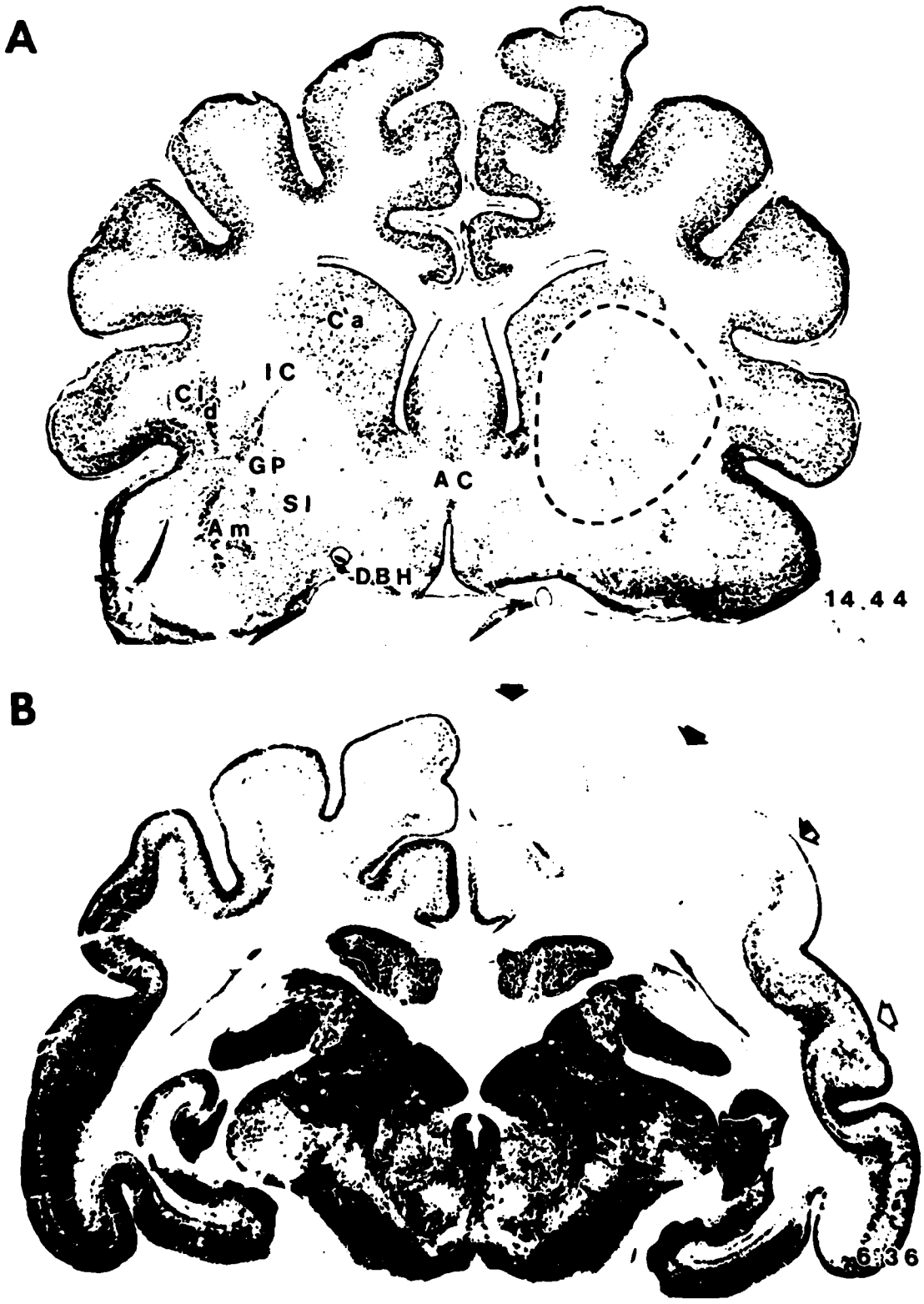
11.44

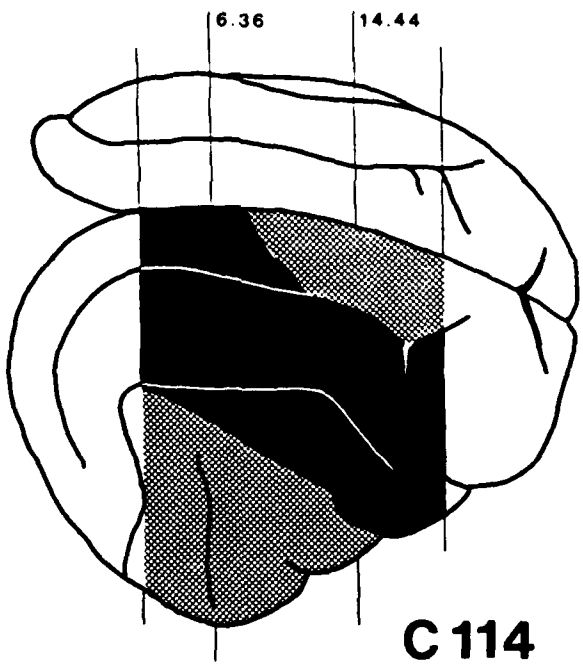

LV
IC
Ca
Put
D
M
L
V
AC
1.0 mm
A
50 µm
B


17.64
16.04
14.44
12.84
Cl$_d$
IC
Ce
LV
Put
NS
SI
AC
OBV
DBH
GP
Am
Cl$_v$
17.64
16.04
14.44
12.84

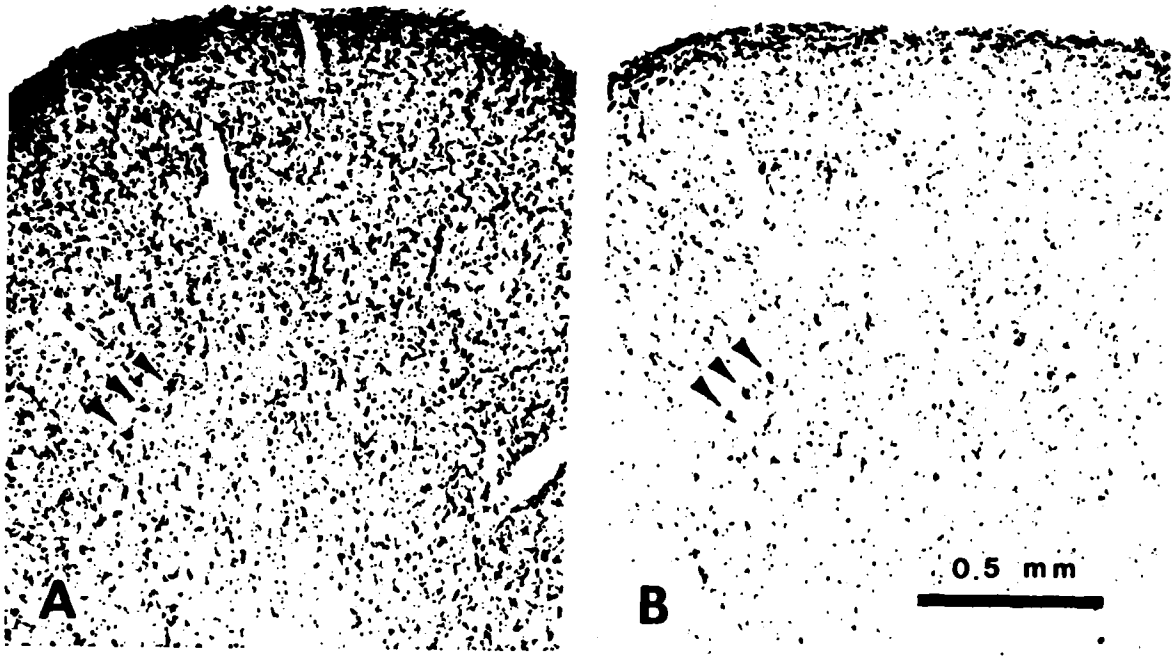
**C 114**

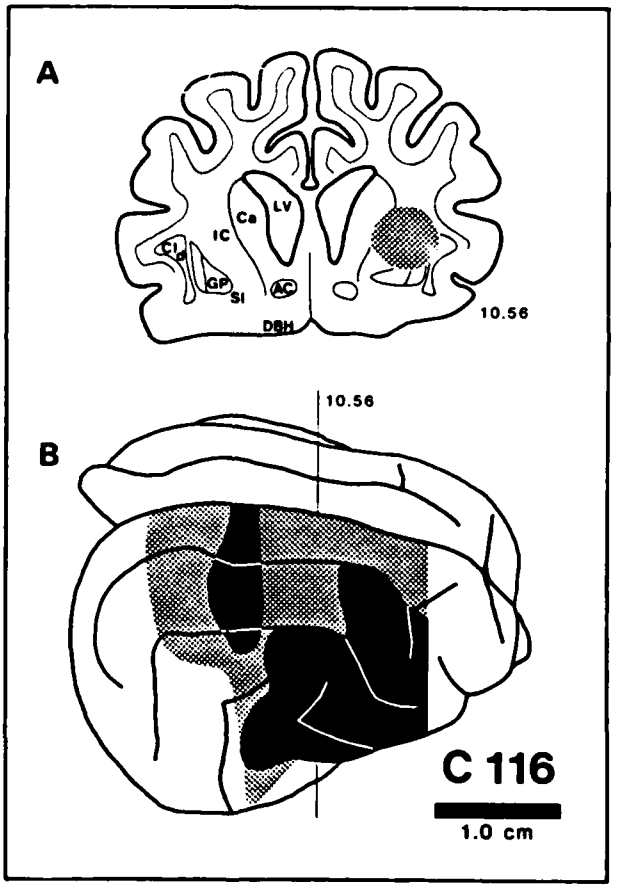
1.0 cm

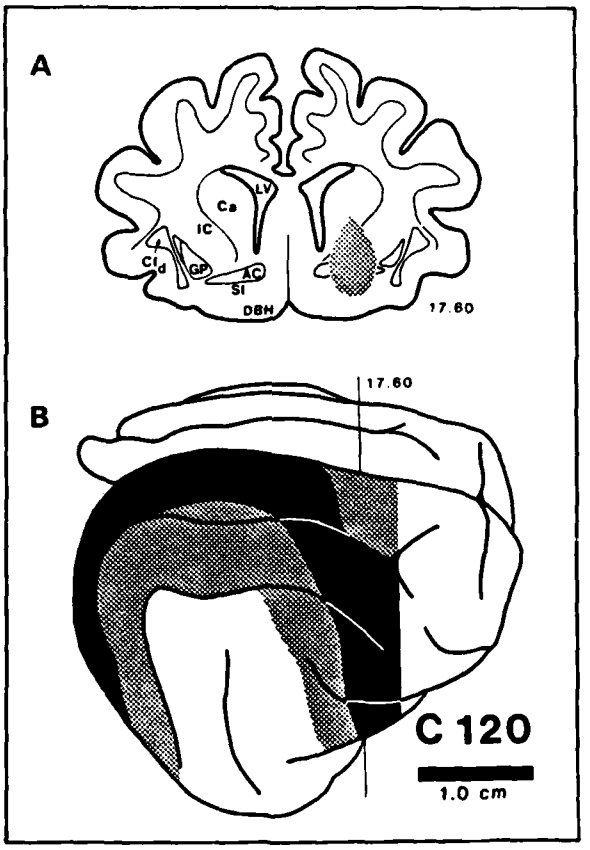

A
Ca
IC
Cl
d
GP
SI
Am
AC
D.B.H
14.44
B
6.36

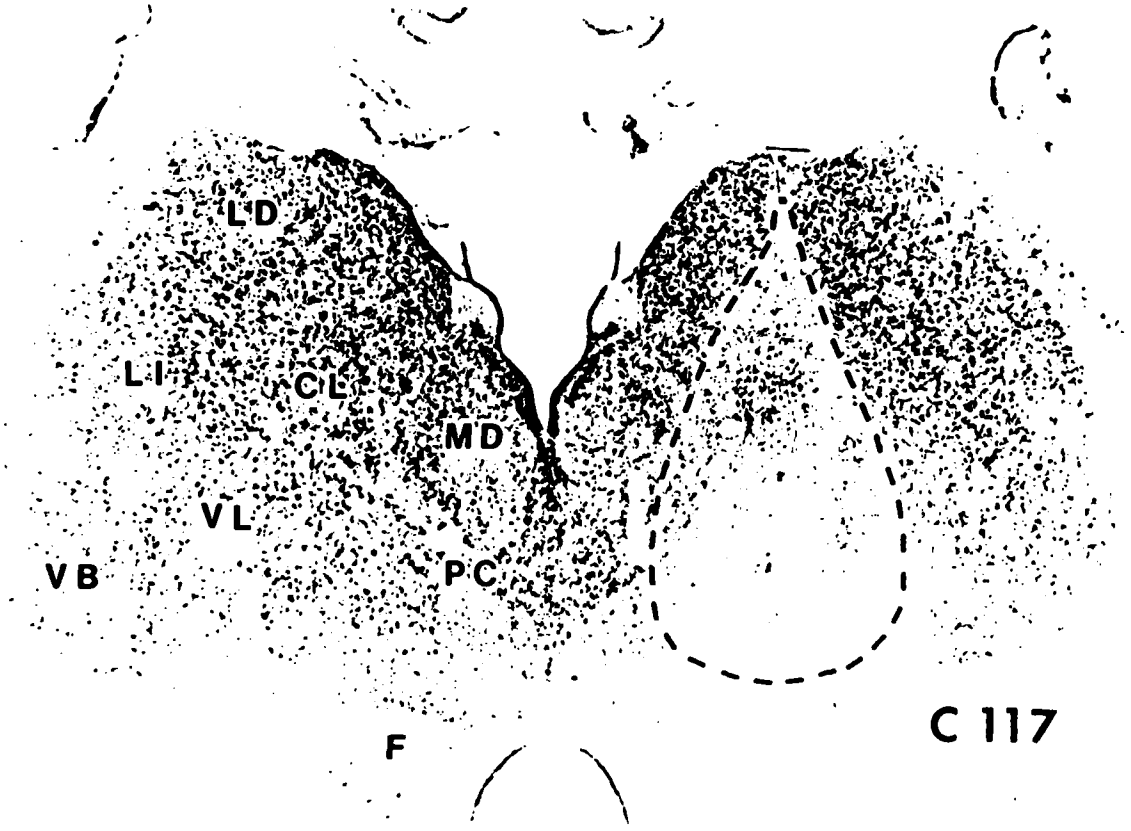


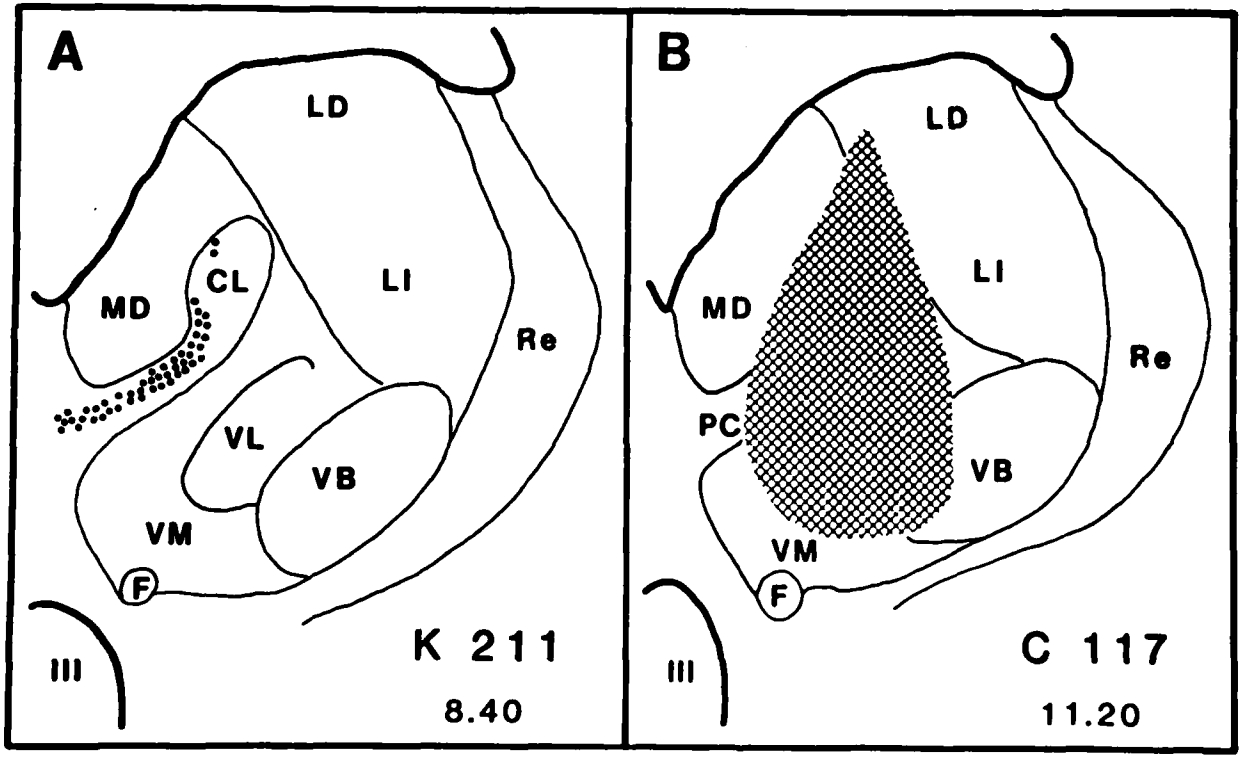

C 114

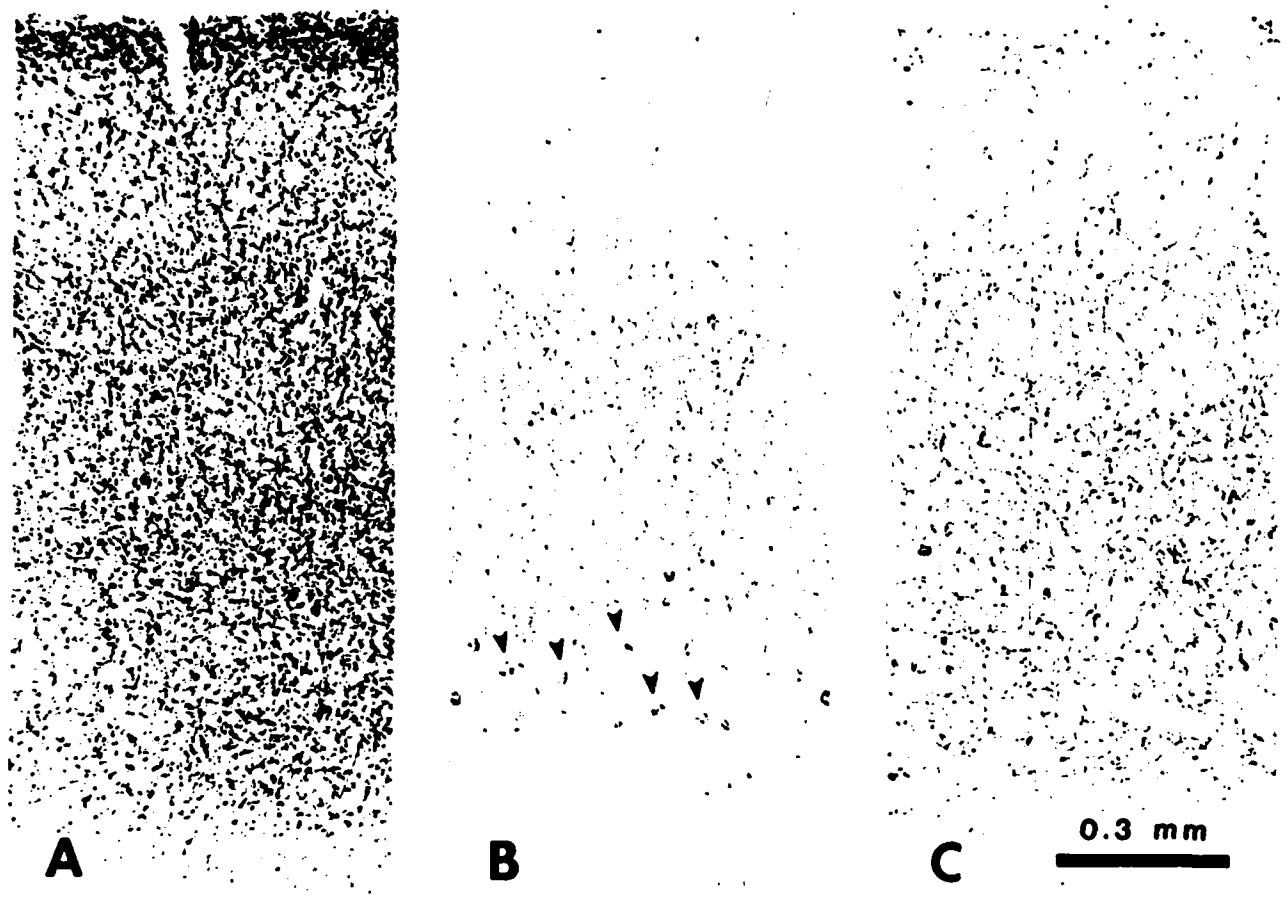

A
B
0.5 mm

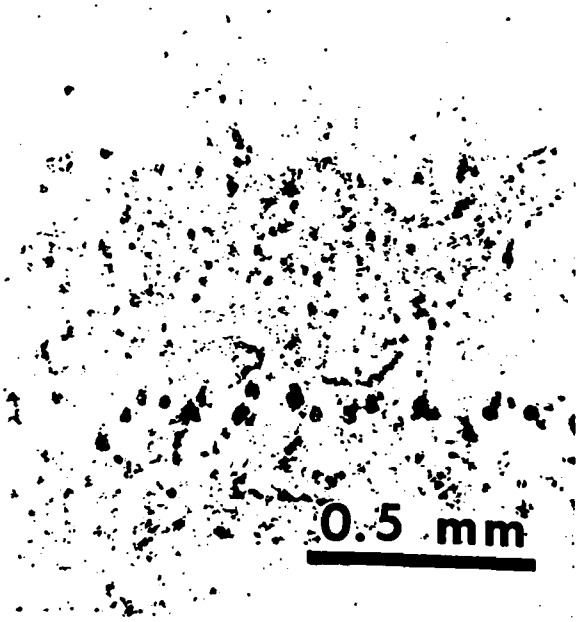

A
Ca
LV
IC
Cl
GP
SI
AC
DBH
10.56
10.56
B
C 116
1.0 cm

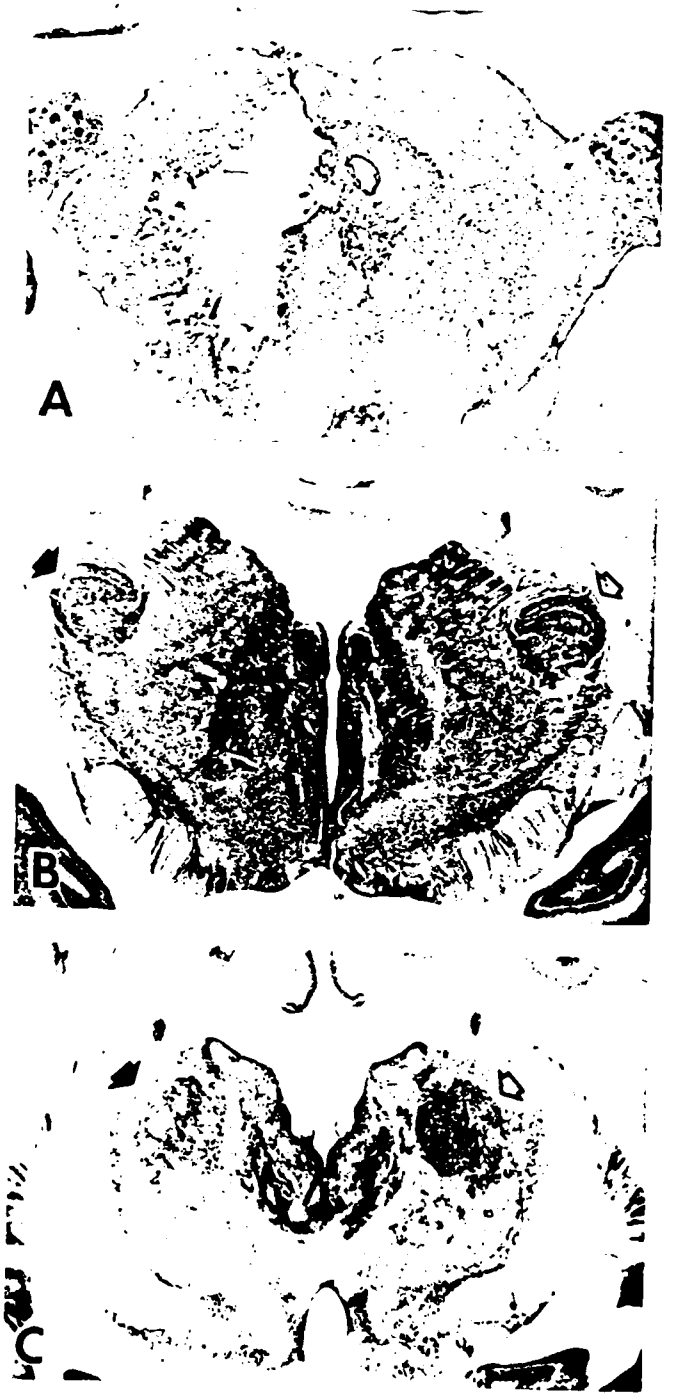

A
Cs
LV
IC
Cl d
GP
AC
SI
DBH
17.60
B
17.60
C 120
1.0 cm


LD
LI
CL
MD
VL
VB
PC
F
C 117


A
LD
CL
LI
MD
Re
VL
VB
VM
F
III
K 211
8.40
B
LD
LI
MD
Re
PC
VB
VM
F
III
C 117
11.20


A
B
C
0.3 mm


0.5 mm


A
B
C